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A Procedure for Gas Chromatographic Analysis of Free Amino Acids in Meats

SUMMARY—A gas liquid chromatography (GLC) procedure was developed to quantitatively measure the free amino acid content of meat and meat products. The amino acids were extracted from the meats with water and purified using dialysis and ion exchange chromatography. n-Butyl N-trifluoroacetyl amino acid derivatives were prepared and analyzed on the gas chromatograph. Factors used for quantitative purposes were calculated using the peak areas and concentrations of known amino acid derivatives.

The isolation and purification procedures were able to remove all carbohydrates, proteins, and 98% of all inorganic salts from the amino acid solutions. Eleven of the common amino acids were completely resolved using the GLC condition described in this investigation. In addition, three GLC peaks contained two amino acids each. The procedure was able to detect free amino acid in sausage in concentrations as low as .01mg/g.

INTRODUCTION

IT HAS BEEN WELL ESTABLISHED that amino acids are important flavor precursors in meat (Batzer *et al.*, 1962; Wood *et al.*, 1957; Wood, 1961; Hornstein *et al.*, 1960b; Macy *et al.*, 1964a). Batzer *et al.* (1960 and 1962) concluded that the amino acids were present in a low-molecular weight glycoprotein that contained glucose as the sugar moiety. When the glycoprotein was heated with inosinic acid, a meaty aroma was observed. Hornstein *et al.* (1960a) identified amino acids and reducing sugars in the water extract of meat, which, upon heating, produced the meaty aroma. Bender *et al.* (1958) exhaustively analyzed the dialyzable water soluble fraction of ox muscle and concluded that it contained primarily amino acids. Wood (1961) made a synthetic amino acid mixture similar in amino acid content to that which Wood *et al.* (1957) had found in their analysis of ox muscle. In addition, Wood found that when the mixture was heated with glucose, a meaty aroma and flavor was produced. Macy *et al.* (1964a) identified 31 amino containing compounds in the water extracts of beef, pork, and lamb muscle. They also demonstrated that large amounts of these compounds were lost and a meaty aroma developed when the extracts were heated (1964b).

In addition to the importance of flavor in amino acids, investigators have used the increase in free amino acids of meats as an index of aging or degree of proteolysis that occur during the storage of meat. Owing to the importance of free amino acids in meats, many investigators have developed qualitative and quantitative procedures for determining them. Awapara (1948) used combinations of aqueous and organic solvents to extract the amino acid from meats and Bender *et al.* (1958), Batzer *et al.* (1960) and Macy *et al.* (1964a) utilized dialysis, filtering, desalting, and lyophilizing techniques to isolate and purify the free amino acids in meats.

Quantitative analysis of amino acids has been accomplished by an amino acid analyzer (Speckman *et al.*, 1958). A semi-quantitative method using paper chromatography to separate the acids and, after treatment with ninhydrin, comparing the density of the resulting spots with those produced by amino acids of known concentration has been used to study the free amino acid content of fermented sausages (Niinivaara *et al.*, 1964).

The analysis of amino acids by gas chromatography (GLC) is a fairly recent innovation. Younger (1959) made the N-acetyl ethyl ester derivatives of eight amino acids and was able to separate them using three different GLC columns. A special method of reacting the amino acids with ninhydrin and then chromatographing the resulting volatile aldehydes was developed by Zlatkis (1960). In 1951, Makesumi *et al.* (1965) prepared the N-trifluoroacetyl methyl esters of the amino acids. By using three gas chromatographs, each operating at a different temperature, they were able to separate all the amino acids. Lamkin *et al.* (1965) were able to quantitatively prepare the derivatives of the common amino acids with the exception of arginine and histidine. Later, Stalling *et al.* (1966) were able to make a derivative of arginine.

The research reported herein was designed to develop a procedure using GLC to determine quantitatively the free amino acids in meat and meat products.

EXPERIMENTAL METHODS

Standard amino acid

Solutions containing the common amino acids were prepared by dissolving 20 mg of each amino acid by dropwise addition of 1 *N* HCl. After the acids were in solution, acidity was adjusted with 0.5 *N* KOH to a pH of 4 to 5. The solution was diluted to 100 ml, and 25 ml were used in the analysis.

Extraction and purification of free amino acids in meat

Two to 3 g of the ground meat sample were extracted overnight at 3°C in the total volume of 80 ml of distilled water. Two drops of chloroform were added as a preservative. The extract was filtered through three layers of cheesecloth and rinsed with 10 ml of water. Then the filtrate was transferred to a dialysis tube and rinsed with another 10 ml of water. The dialysis tube was placed in a 1-liter flask along with 500 ml of distilled water, and the sample was dialyzed for 24 hr at 3°C. Then the diffusate was passed through an ion exchange resin column contain-

ing 30 g of Amberlite IR-120. After the carbohydrates and a portion of the salt had passed through the column, the amino acids were eluted with 150 ml of 2 *N* NH_4OH. The amino acid solution was concentrated to 15 ml using a rotary evaporator and a 60°C water bath. After concentration, the sample was desalted on a RSC model 1930 desalter. The voltage was set at 40, and the sample was desalted until only 0.2 milliamps would pass through the sample. After desalting, the internal standard norleucine was added, and the sample was completely dried on a rotary evaporator using a 60°C water bath.

The ion exchange resin was regenerated by passing 100 ml of 1 *N* HCl through the column After washing with 150 ml of distilled water, the resin was ready to be used in another analysis.

Formation of derivatives

N-Butyl N-trifluoroacetyl derivatives of the amino acids, isolated according to the procedure just mentioned, were made using the procedures of Lamkin *et al.* (1965) and Stalling *et al.* (1966). After esterification, the derivatives were dissolved in chloroform and transferred to a small vial, concentrated to a volume of 0.5 ml and analyzed by GLC. An F & M model 810 gas chromatograph equipped with dual hydrogen flame detectors was used for the analysis. The GLC conditions were as follows:

Column: 1% neopentylglycol succinate on 60–80 mesh chromosorb W
Column size: ¼ in. × 4 ft
Oven temperature: 100°C for 5 min and programmed at a rate of 4°C/min to 218°C
Injector temperature: 230°C
Detector temperature: 290°C
Carrier gas and flow rate: Helium 50 ml/min
Hydrogen flow rate: 54 ml/min
Air flow rate: 333 ml/min

Quantitative factors

The calibration of the detector and recorder system used for quantitative analysis is a necessary part of the analysis. This was done by calculating factors that related the peak area per weight of internal standard to the peak area per weight of amino acids to be analyzed. During the determination of these factors, 25 ml of the standard amino acid solution, including the internal standard (norleucine), were carried through the entire procedure of isolation, purification, derivative formation, and GLC analysis. An appropriate factor for each acid was calculated from the resulting peak areas of the n-butyl N-trifluoroacetyl derivatives by the following equation:

$$\text{Factor} = \frac{\text{wt amino acid} \times \text{measured area of norleucine peak}}{\text{measured area of amino acid peak} \times \text{wt of norleucine added to the sample}}$$

RESULTS AND DISCUSSION

Fig. 1 shows a gas chromatogram of the 17 standard amino acids used in this investigation. There was complete separation of 11 of these amino acids. Three sets of two amino acids each could not be separated using the conditions described herein. These include threonine and leucine, proline and serine, and aspartic acid and phenylalanine.

Table 1. Identification of peaks and retention times (min).

1	chloroform (solvent)	
2	alanine	9.2
3	valine	10.4
4	isoleucine	12.5
5	glycine	13.2
6	threonine + leucine	14.4
7	norleucine	15.0
8	proline + serine	18.0
9	hydroxyproline	22.0
10	methionine	24.0
11	aspartic acid + phenylalanine	25.0
12	glutamic acid	29.0
13	tyrosine	33.0
14	lysine	36.5
15	tryptophan	39.2

Chromatograms such as this were used to calculate the quantitative factors for individual amino acids listed in Table 2. Although two methods of acylation were used, the factors were essentially identical. After the factors were calculated, the equation was rearranged and used to determine the weight of amino acids in the samples being analyzed.

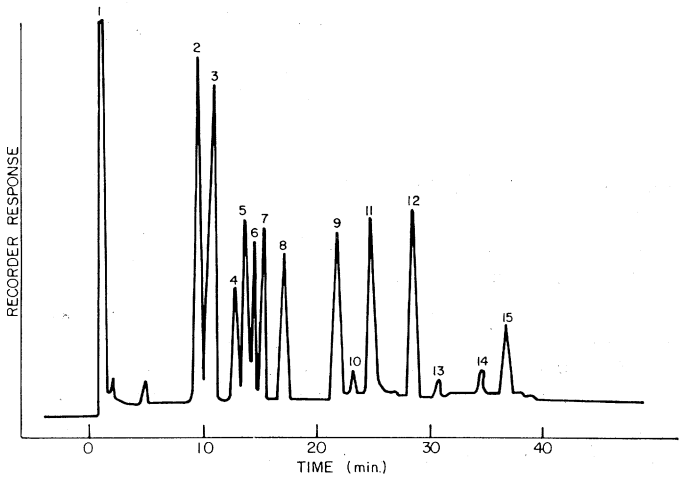

Fig. 1. Chromatogram of 17 standard amino acids. Numbered peaks are identified in Table 1.

Table 2. Factor and the standard deviation for each amino acid.[1]

alanine	1.317	0.177
valine	1.430	0.180
isoleucine	1.406	0.226
glycine	1.291	0.260
threonine + leucine	1.692	0.217
proline + serine	1.813	0.208
hydroxyproline	1.714	0.253
methionine	2.097	0.403
aspartic acid + phenylalanine	1.003	0.171
glutamic acid	1.399	0.190
tyrosine	12.007	1.382
lysine	11.307	1.197
tryptophane	1.457	0.157

[1] average of 8 determinations.

Table 3. Analysis of a known amino acid mixture.[1]

Amino acid	1	2	3	Average value	True value	Relative percent error
Alanine	2.96	2.86	2.85	2.89	2.90	0.34
Valine	2.08	1.97	2.11	2.05	1.98	3.50
Isoleucine	2.86	2.75	2.50	2.70	2.35	14.90
Glycine	4.40	4.03	4.85	4.42	4.82	8.29
Threonine + leucine	3.32	2.95	3.10	3.12	2.85	9.47
Proline + serine	4.56	4.38	3.99	4.31	4.40	2.04
Hydroxyproline	2.70	2.62	2.86	2.72	2.30	18.30
Aspartic acid + phenylalanine	3.09	3.48	3.60	3.42	3.05	12.13
Glutamic acid	2.18	2.22	2.34	2.74	2.32	3.57
Tyrosine	3.70	4.07	4.81	4.20	3.80	10.52
Lysine	3.35	2.90	2.49	2.91	2.85	2.10
Tryptophan	3.34	2.50	2.74	2.86	3.10	7.74

[1] Mg of free amino acid/sample.

To determine the accuracy of the procedure, samples with known amounts of amino acids were analyzed. Table 3 shows the results of this experiment. The relative percent error in most cases was below 10%. Other samples were analyzed using varied amounts of amino acids and essentially the same results were obtained.

We found in preliminary work that some water soluble carbohydrates as well as amino acids were extracted from the meat. The presence of carbohydrates caused a brown color to develop during the evaporation and drying steps in the procedure. We concluded that the free amino acids were reacting with the carbohydrates in a nonenzymatic browning reaction. This reaction caused a loss of amino acids and also resulted in the formation of a black tar-like residue during the acylation of the amino acids. The carbohydrates were removed by using an ion exchange resin, and the browning reaction was eliminated.

Meat extracts also contained excess salts that interfered with acylation of the amino acids. This salt was removed by the use of a laboratory desalting unit. Experiments were conducted using known weight of amino acids, glucose, and salts to determine if amino acids were lost in these steps. The results indicated that no loss of amino acids occurred during the removal of carbohydrates and salt from the sample. All carbohydrates and 20% of the salt were removed from the amino acid extracts with the ion exchange column. After desalting, 98.5% of the salt had been removed from the samples. The remaining salt, which was found to be NH_4Cl, could not be removed by the desalter. However, this small amount of NH_4Cl did not affect the formation of derivatives, and so its presence was ignored.

The procedure was used to measure the free amino acids in country cured hams, dry cured sausages, and fresh beef. Only the data for sausages is given herein to demonstrate the GLC procedure for measuring free amino acids.

Sausage analysis. The GLC procedure was used to measure the free amino acid content of some salami type sausages. Fig. 2 shows a typical chromatogram obtained from a mature Genoa sausage. The graph is similar to that obtained for the standard amino acids (Fig. 1).

The free amino acid contents of 5 sausages (1 fresh and 4 mature) are listed in Table 4. The total free amino

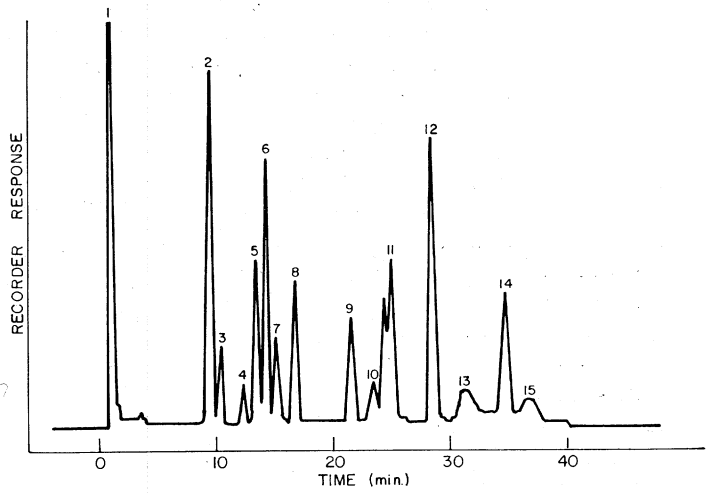

Fig. 2. Chromatogram of a mature sausage. Numbered peaks are identified in Table 1.

acids ranged from 2.26 mg/g in the fresh sausage to approximately 20 mg/g in the fully aged, mature sausages. Two of the amino acids were found in .01 mg/g quantities, indicating that the procedure is capable of detecting small concentrations of free amino acids in meat.

The procedure described herein has proven a reliable way to measure the degree of proteolysis occurring during the aging and curing of meat and meat products. Although considerable time is involved in the necessary purification of the amino acids before acylation, several samples can be analyzed simultaneously, thereby reducing the time required per analysis. Further work needs to be done to develop a GLC column that is capable of separating all amino acid derivatives.

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Table 4. Free amino acid content of one raw and four finished Genoa Sausages manufactured on different days.[1]

Amino acid	Raw sausage	Finished sausage 1[2]	2[3]	3[3]	4[3]
Alanine	0.54 ± .12[4]	1.73 ± .16	1.82 ± .30	2.43 ± .32	1.93 ± .02
Valine	0.09 ± .02	0.83 ± .03	0.54 ± .07	0.86 ± .22	0.48 ± .03
Isoleucine	0.34 ± .05	0.44 ± .03	0.31 ± .09	0.52 ± .03	0.20 ± .02
Glycine	0.18 ± .05	0.93 ± .12	0.78 ± .05	0.90 ± .10	0.98 ± .06
Threonine + leucine	0.11 ± .05	1.82 ± .09	1.96 ± .51	2.77 ± .41	1.53 ± .05
Proline + serine	0.09 ± .03	1.42 ± .08	1.05 ± .07	1.31 ± .02	1.03 ± .02
Hydroxyproline	0.01 ± .01	0.43 ± .07	0.45 ± .13	1.45 ± .15	0.60 ± .28
Methionine	0.01 ± .01	0.15 ± .15	0.29 ± .07	0.45 ± .09	0.37 ± .01
Asparic acid + phenylalanine	0.10 ± .01	1.20 ± .09	1.17 ± .17	1.62 ± .06	1.36 ± .06
Glutamic acid	0.48 ± .09	1.51 ± .37	1.61 ± .07	2.12 ± .01	1.98 ± .20
Lysine	1.20 ± .30	9.84 ± .83	10.13 ± 3.20	13.81 ± 1.32	8.32 ± 2.20
Tryptophan	0.11 ± .04	0.11 ± .02	0.11 ± .01	0.10 ± .01	0.16 ± .03
Total	2.26	20.41	18.60	28.34	18.94

[1] Mg of free amino acid/gram of sausage.
[2] Triplicate analysis.
[3] Duplicate analysis.
[4] Average deviation.

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